Bosphore® HCV Genotyping Kit v1

USER MANUAL

For *in vitro* Diagnostic Use

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1. PRODUCT DESCRIPTION

Bosphore® HCV Genotyping Kit v1 detects and characterizes the genotype of Hepatitis C Virus in human serum, encompassing 4 major and most predominant HCV genotypes (1,1a,1b,2,3,4). The analytic sensitivity is 100 IU/ml. A region within the 5'UTR is amplified and fluorescence detection is accomplished using the FAM filter.

An internal control has been integrated into the kit in order to check PCR inhibition. The amplification data of the internal control is detected with the Cy5 filter. The internal control can be added either during RNA extraction or PCR step.

2. CONTENT

Bosphore® HCV Genotyping Kit v1 is composed of Real-Time RT PCR reagents and positive and negative controls:

Component	REAGENT	100 Tests	50 Tests	25 Tests
1	dH_2O	(1000 µl)	(1000 µl)	(500 µl)
2	PCR Mix	(4400 µl)	(2200 µl)	(1100 µl)
3	RT Mix	(44 µl)	(22 µl)	(11 µl)
4	Detection Mix1	(616 µl)	(308 µl)	(154 µl)
5	Detection Mix2	(616 µl)	(308 µl)	(154 µl)
6	Detection Mix3	(616 µl)	(308 µl)	(154 µl)
7	Detection Mix4	(616 µl)	(308 µl)	(154 µl)
8	Internal Kontrol	(560 µl)	(280 µl)	(140 µl)
9	Positive Control 1	(120 µl)	(60 µl)	(30 µl)
10	Positive Control 2	(120 µl)	(60 µl)	(30 µl)
11	Positive Control 3	(120 µl)	(60 µl)	(30 µl)
12	Positive Control 4	(120 µl)	(60 µl)	(30 µl)

3. STORAGE

Bosphore® HCV Genotyping Kit v1 PCR reagents should be stored at -20°C. Repeated thawing and freezing (>3x) should be avoided since it may reduce sensitivity. If the components are to be used in small amounts, they should be frozen in aliquots.

While preparing the PCR; the components should not be exposed to room temperature for more than 10 min. and the detection mix components should not be exposed to light more than 1-2 min. We recommend preparing the PCR on a cooling block and keeping the detection mixes within a closed container.

The components maintain their stability until the expiry dates on the labels, if they are stored at advised conditions.

4. REQUIRED MATERIALS AND DEVICES

- Montania® 483 Real-Time PCR Instrument (Anatolia Geneworks), or another Real-Time PCR system with FAM, HEX and Cy5 filters (iCycler, iQ5, CFX–BioRad, LightCycler 1.5, 2.0, 480-Roche, 7500 Real-Time PCR System-ABI, Stratagene Mx3005P, Mx3000P-Agilent, LineGeneK, LineGene 9600-Bioer, Rotorgene 2000, 3000, 6000, Q-Qiagen)
- 0.2 ml Thin-Wall PCR tubes or strips
- Magnesia® 16 Nucleic Acid Extraction System / Magnesia® Viral Nucleic Acid Extraction Kit (Anatolia Geneworks), or other high quality viral RNA extraction kits and systems
- Deep freezer (-20°C)
- Desktop centrifuge with rotor for 2 ml. microcentrifuge tubes
- Calibrated adjustable micropipettes

- DNAse, RNAse, pyrogen free micropipette tips with filters
- DNAse, RNAse, pyrogen free 1.5 or 2 ml. microcentrifuge tubes
- Disposable laboratory gloves

5. IMPORTANT NOTES AND SAFETY INSTRUCTIONS

Important!:

- The product should be delivered on dry ice. Check for presence of dry ice upon arrival.
- Check for the expiry dates on the box and tube labels, upon arrival. Do not use expired products or components.
- Calibrated or verified micropipettes, DNAse, RNAse, pyrogen free micropipette tips with filters, and DNAse, RNAse, pyrogen free microcentrifuge tubes should be used.
- Before starting a test procedure, all components should be thoroughly thawed. After thawing, all components should be centrifuged briefly (spin-down for 3-5 seconds), and mixed well to ensure homogeneity prior to use.
- The kit components should be kept on ice or a cooling block until the reaction is prepared, and they should be quickly returned to -20ºC.
- PCR and nucleic acid isolation must be performed in different compartments. Samples should be stored separately to avoid contact with the kit components.
- Pathogen information should be reviewed to be aware of the health related risks.
- Serum samples should be handled with extreme caution, suitable class microbiological safety cabinet should be used: Physical contact with pathogens should be avoided by; wearing lab coats and gloves, no allowance for eating or drinking within the workspace, prevention of unauthorized individuals' access to the working area.
- All the pathogenic wastes produced during the nucleic acid isolation step; including the serum samples and material contacted with them, should be discarded into medical waste and disposed safely.

6. PRODUCT USE LIMITATIONS

- All the components may exclusively be used for in vitro diagnostics.
- This product should be used in accordance with this user manual, by personnel specially trained to perform in vitro diagnostic procedures.

7. PATHOGEN

Causative Agents

The hepatitis C virus is a hepacivirus of the Flaviviridae family of viruses that causes Hepatitis C in humans. It is a small, enveloped, 9.6kb single-stranded RNA virus that is classified into six main genotypes (1-6) with more than one hundred different subtypes. (1)

Genotype 1 is the most common one and is the one with the least response to therapy. Since HCV has a high tendency to mutate, and doesn't initiate a severe response in human T- lymphocytes of the immune system (a white blood cell type), it results a high rate of chronic infection. The genetic heterogeneity of this virus, which cannot be

grown by cell culture, makes the diagnosis difficult, lowers the response to treatment and also impedes the development of the vaccine against the disease(4,5). It has been observed that different HCV genotypes show different responses to antiviral therapy. The duration and success rate of HCV medication (PEG-IFN and ribavirin) mostly depend on the virus genotype. The response rate to treatment in genotype 2 and 3 is higher than the one in genotype 1 and 4 (70-80% against 40-50% in long term). Moreover, the successful treatment of genotype 2 and 3 takes approximately 6 month, while it is 1 year for genotype 1 and 4 (6,7,8). It has been reported that the response to treatment is 0-3%, and ceasing the treatment should be considered, if the HCV RNA level of the patients has not revealed at least 2 log decrease after the 12 weeks of treatment (9, 10). It has been observed that duration of the treatment of acute infection of genotype 1 is shorter and the success rate is higher than the chronic infection of genotype 1 (11, 12, 13).

The nucleotide sequences of genotypes differ around 31-34% from each other, the subtypes differ around 20 to 23%. Though the genotypes first appeared endemically in geographically distinct regions, currently they are spread all over the world. As the Genotypes 1, 2 and 3 are widely seen all over the world, genotype 4 and 5 are predominant in Africa. For instance, in the U.S., approximately, 75% of all cases are caused by genotype 1, 15% by genotype 2, 5% by genotype 3 and 1% by genotype 4. Genotype 6 is typical to Southeast Asia, genotype 1 is prevalent in Western Europe and U.S, genotype 3 is very common in the UK. (14, 15, 16)

Epidemiology

It is estimated that HCV has a worldwide prevalence of 3% affecting around 180 million people with between 3 to 4 million new infections each year. The vast majority of infected people (70-90%) develop chronic infection. Though chronic infection may be asymptomatic, it is a leading cause of chronic liver diseases, including cirrhosis in between 20 to 50% of patients. Treatment may be effective in 10-50% of patients depending on the applied therapy. (2)

Modes of Transmission:

Hepatitis C is believed to be spread through contact with infected blood. However, unlike many other blood borne viruses, HCV may be transmitted even through indirect sources like a used razor, making HCV more transmissible than other blood borne viruses –including HIV. Common routes of transmission include transfusion of blood products, intravenous and percutaneous drug and needle use, work accidents among healthcare workers and any other blood to blood contacts, such as sexual practices and from mother to newborn (maternal-infant transmission). Statistical studies have revealed no risk factors for HCV transmission in the activities of daily living (sneezing, coughing, hugging, etc.). (2), (3)

8. METHOD

Bosphore® HCV Genotyping Kit v1 is based on the Real Time RT PCR method. HCV genetic material is amplified by reverse transcription technique since it is composed of RNA. RT-PCR, which is also referred as RNA PCR, is a two-step reaction. First, complementary DNA is synthesized from RNA by reverse transcription and then complementary DNA is amplified by standard PCR. The primer binds to the target RNA region in RT-PCR and RNA-DNA double strand is synthesized by reverse transcriptase enzyme using the RNA template for complementary DNA. Afterwards, standard PCR continues.

Polymerase chain reaction is a technique that is used for amplification of a DNA region. The reaction occurs by the repeating cycles of heating and cooling. The main components of PCR are primers, dNTPs, Taq polymerase enzyme, buffer solution and template. As a brief explanation, primers are small synthetic DNA those anneal to the

specific regions of the template in order to start the synthesis. dNTPs are the building blocks of the amplified products. Taq polymerase amplifies the DNA template. Buffer solution provides the pH adjustment required for the reaction and template, as referred, is the target region for synthesis. In addition to these components, in RT PCR reverse transcriptase is added to the reaction and cDNA synthesis from the RNA template is acquired.

In Real Time PCR technique, in contrast to conventional PCR, PCR product can be monitored during the reaction. Therefore Real-Time PCR obviates the need for further analysis methods like gel electrophoresis, whereby minimizing the risk of contamination. Dual labeled probes employed in the reaction in addition to the conventional PCR reagents, enable detection of the amplified target with increased sensitivity. I

The assay utilizes the 5' exonuclease activity of Taq Polymerase to cleave a dual-labeled fluorescent hydrolysis probe during the extension phase of PCR.

The probe is labeled at the 5' end with a fluorescent 'reporter' molecule, and at the 3'end with another fluorescent molecule that acts as a 'quencher' for the 'reporter'. When the two fluorophores are in close proximity, and the reporter is excited by light, no reporter fluorescence can be detected. During the elongation step of PCR, Taq Polymerase encounters and cleaves the probe bound to the template. As the reporter is freed from the suppressing effect of the quencher, fluorescence signal can be detected.

The fluorescence generated by the reporter increases as the PCR product is accumulated; the point at which the signal rises above background level and becomes distinguishable, is called the threshold cycle (C_T). There is a linear relationship between the log of the starting amount of a template and its threshold cycle, thus starting amount of unknown templates can be determined using standard curves constructed using C_T values of the known starting amounts of target templates.

Bosphore® HCV Genotyping Kit v1 employs multiplex PCR, and an internal control is incorporated into the system in order to control the isolation procedure and to check for possible PCR inhibition. HCV RNA (cDNA) and an internal control are co-amplified in a single reaction, using sequence-specific primers. The fluorescent signal generated by the HCV amplification is detected by a probe labeled at the 3' end with FAM, through the FAM channel. The fluorescent signal generated by the internal control amplification, is detected by a second probe (labeled at the 5' end with a different reporter molecule, Cy5) through the Cy5 channel.

9. PROCEDURE

9.1. Sample Preparation, Storage and Transport

To isolate serum from the clinical specimen, the blood sample should be collected into sterile vacutainers without any anticoagulant. For venipuncture, only sterile material should be used.

Attention: EDTA or heparin containing vacutainers should not be used for sample collection.

The serum should be separated from blood within 6 hours after blood collection. To separate the serum, the blood container should be centrifuged at 800-1600 x g for 20 minutes. The separated serum should be transferred to polypropylene tubes and stored at -20°C or lower, until use.

The samples should be transported in containers with capacity to resist pressure. Transportation should be done according to local and national regulations for pathogen material transport.

9.2. Interfering Substances

To avoid possible influences on PCR:

- Hemolytic samples,

- Samples of heparinized patients,
- Samples of patients with elevated levels of bile salts, bilirubin or lipids

must not be used.

9.3. RNA Isolation

We recommend that the Magnesia® 16 Nucleic Acid Extraction System / Magnesia® Viral Nucleic Acid Extraction Kit (Anatolia Geneworks) isolation system is used with Bosphore® HCV Genotyping Kit v1. The RNA isolation should be performed according to the manufacturers' instructions. The recommended starting volume is 400 µl and the elution volume is 60 µl. The amount of internal control that should be used during isolation for each system is 5 µl.

9.4. Kit Components

9.4.1. PCR Mix

PCR mix contains; HotStarTaq DNA Polymerase, Probe RT-PCR Buffer, and ROX passive reference dye.

HotStarTaq DNA Polymerase: HotStarTaq DNA Polymerase is a modified form of Taq DNA Polymerase, and is provided in an inactive state and has no enzymatic activity at ambient temperature. The enzyme remains completely inactive during the reverse-transcription reaction and does not interfere with it. This prevents formation of misprimed RT-PCR products and primer–dimers during reaction setup, reverse transcription, and the first denaturation step. The enzyme is activated after the reverse-transcription step by a 15-minute, 95°C incubation step. The hot start also inactivates the reverse-transcription enzymes, ensuring temporal separation of reverse transcription and PCR, and allowing both steps to be performed sequentially in a single tube.

Probe RT-PCR Buffer: It is a unique OneStep RT-PCR buffer system, and has been specifically adapted for realtime RT-PCR using sequence-specific probes. The buffer contains a balanced combination of KCl and (NH4)2SO4.

ROX passive reference dye: For certain real-time cyclers, the presence of ROX passive reference dye in real-time PCR compensates for non-PCR–related variations in fluorescence detection. Fluorescence from ROX dye does not change during the course of real-time PCR, but provides a stable baseline to which PCR-related fluorescent signals are normalized. Thus, ROX dye compensates for differences in fluorescence detection between wells due to slight variations in reaction volume or to differences in well position. The use of ROX dye is necessary for all instruments from Applied Biosystems and is optional for instruments from Stratagene (e.g., Mx3000P®, Mx3005P®, and Mx4000®). Montania 483, Rotor-Gene cyclers and instruments from Bio-Rad/MJ Research, Cepheid, Eppendorf, and Roche do not require ROX dye. The presence of ROX dye in the master mix does not interfere with real-time PCR on any instrument, since the dye is not involved in the reaction and has an emission spectrum completely different from fluorescent dyes commonly used for probes.

9.4.2. RT mix

RT Mix contains a unique Omniscript and Sensiscript blend. Both enzymes exhibit a high affinity for RNA, facilitating transcription through secondary structures that may inhibit other reverse transcriptases. Omniscript is designed for reverse transcription of RNA amounts greater than 50 ng, and Sensiscript is optimized for use with very small amounts of RNA (<50 ng). This enzyme combination provides highly efficient and sensitive reverse transcription over a wide range of RNA template amounts.

9.4.3. Detection Mix 1

Detection Mix 1 contains forward and reverse primers and dual-labeled probes specific for HCV genotypes 1b and 4 and for internal control.

9.4.4. Detection Mix 2

Detection Mix 2 contains forward and reverse primers and dual-labeled probes specific for HCV genotypes 1a and 2 and for internal control.

9.4.5. Detection Mix 3

Detection Mix 3 contains forward and reverse primers and dual-labeled probes specific for HCV genotype 3 and for internal control.

9.4.6. Detection Mix 4

Detection Mix 4 contains forward and reverse primers and dual-labeled probes specific for HCV genotype 1 (1 c, d, e, f, g, h, i, j, k) and for internal control.

9.4.7. Internal Control

An internal control is included in the kit to control RNA isolation and PCR inhibition. The internal control is a synthetic DNA molecule derived from human genome. It is added into the serum, proteinase K and carrier RNA mixture during DNA isolation, to control the isolation efficiency and PCR inhibition. The amount of IC that should be added during isolation is 5 µl per 400 µl serum sample. Alternatively, the internal control can be added directly into the PCR master mix to control the PCR inhibition exclusively. For this purpose, 0.1 µl of internal control should be added for each reaction into the master mix. **Caution: It is not necessary to include the internal control in the reaction if it has already been added during the extraction step.** Lack of internal control amplification in the FAM negative samples, may indicate a problem in isolation or PCR inhibition. In this case, isolation and PCR should be repeated. In samples that contain a high viral load, internal control can be suppressed and no increase of the signal is detected. Please use the table below for the interpretation of internal control data:

HCV(FAM)	Internal Control (Cy5)	Interpretation
+	+	Sample positive
-	+	Sample negative
+	-	Sample positive
-	-	Repeat the test!

9.4.8. Positive Control

The kit includes 4 positive controls calibrated with the International WHO Standard (NIBSC Code 06/100); HCV 1b RNA, HCV 1a RNA, HCV3 RNA and HCV1 (1 c, d, e, f, g, h, i, j, k) RNA and each positive control should be used with its specific Detection Mix; Detection Mix 1, 2, 3 and 4; respectively. It must be included in the PCR to test the efficiency of the PCR exclusively.

9.5. Preparing the RT-PCR

Make sure that all the kit components are thawed before use. Refer to the table below for preparing the PCR. It is for only one reaction, multiply these values with the sample number to find the values required for the master mix. While preparing master mixes for more than 5 samples, an extra 10% should be added to the total sample number.

PCR Mix	20 µl
RT-mix	0.4 µl
Detection Mix	5.6 µl
Internal Kontrol	0.1 µl*
Sample RNA	14 µl
(Negatif/Pozitif Control)	
Total Volume	40 µl

*** It is not necessary to include the internal control in the reaction if it has already been added during the extraction step.**

Pipette 26 µl of the master mix into the PCR tubes or strips, and add 14 µl of RNA (sample/ positive or negative control). Close the tube cap. Make sure that the solution in each tube is at the bottom of the tube. Centrifuge if necessary.

9.6. Programming the Montania® 483 Real-Time PCR Instrument

The thermal protocol for Bosphore® HCV Genotyping Kit v1 is composed of two-steps; firstly a reverse transcription step and secondly Real Time PCR steps; an initial denaturation for activation the HotStarTaq DNA Polymerase, a two-step amplification cycle and a terminal hold. The real-time data is collected at the second step of the amplification cycle.

The thermal protocol required to run with the Detection Mix 1, 2 and 3 is in the table below:

Reverse Transcription	50°C	30:00 min.	
Initial denaturation	95°C	14:30 min.	
Denaturation	97°C	00:30 min.	50 cycles
Annealing (Data Collection)	54°C	01:20 min.	
Synthesis	72°C	0:15 min.	
Hold	22°C	05:00	

The thermal protocol required to run with the Detection Mix 1, 2 and 3 is in the table below:

Reverse Transcription	50°C	30:00 min.	
Initial denaturation	95°C	14:30 min.	
Denaturation	97°C	00:30 min.	50 cycles
Annealing (Data Collection)	58°C	01:10 min.	
Hold	22°C	05:00	

Before starting a Real-Time PCR reaction using the Bosphore® Kits, the following steps should be completed:

- Choose the filter pairs to be used (FAM, HEX and Cy5),
- Identify unknown samples, positive and negative controls,
- Select the correct thermal protocol.

These steps are described below:

From the main menu of the Montania® 483 Real-Time PCR Instrument, "File" and then "New" is selected. "Create a new Experiment" is selected. In the "Select Channel" window channels 1 (FAM), 2 (HEX) and 3 (Cy5) are selected (Fig. 1). Samples, positive and negative controls are identified in the "Module Edit" menu (Fig. 2). To select the thermal protocol "Gene Amplification" menu is used. The "Open" button in the "Experiment Program" is clicked and the appropriate thermal protocol is selected. (Fig. 3a). The thermal cycles of the selected protocol is displayed. The experiment starts by clicking the "Start" button (Fig. 3b).

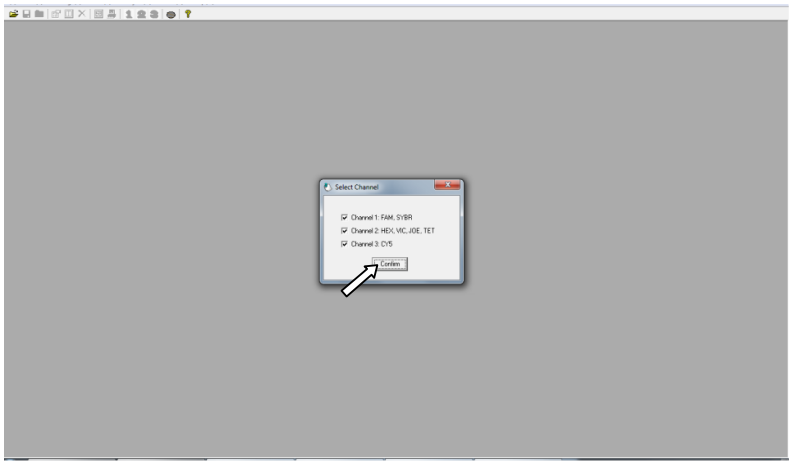

Fig. 1: Filter Selection in Montania® 483

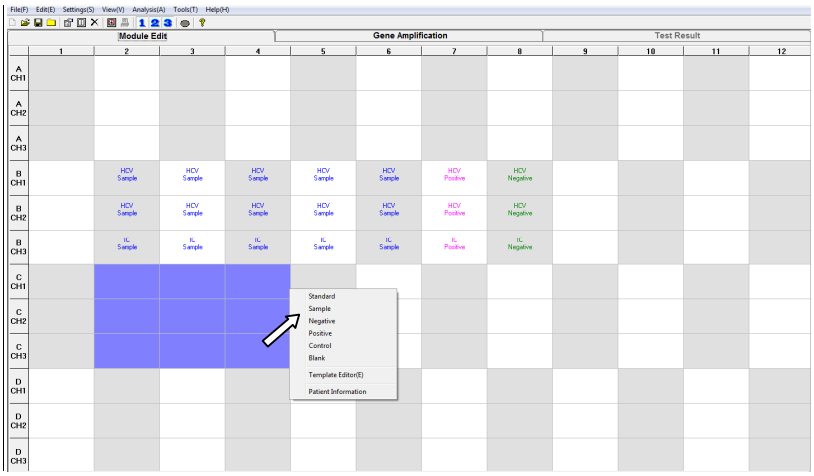

Fig. 2: Sample Location and Identification

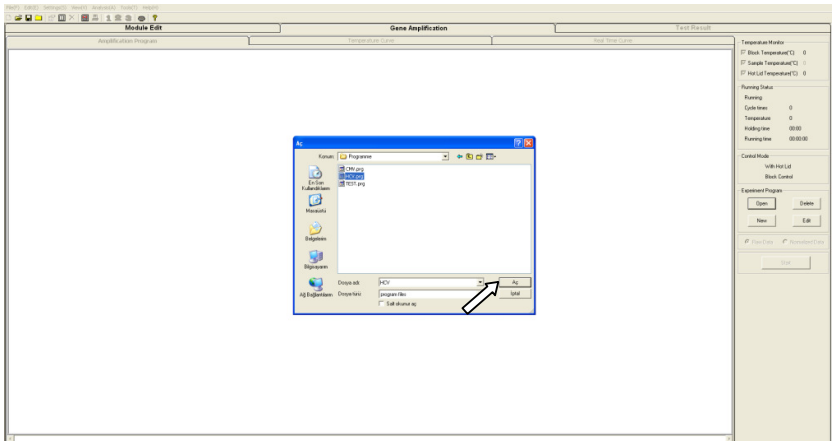

Fig. 3a: Selecting the Thermal Protocol

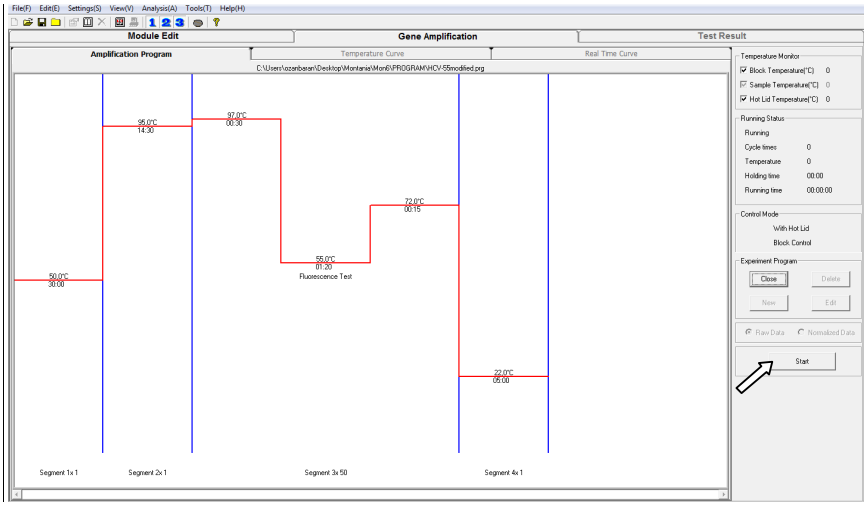

Fig. 3b: Starting the Experiment

10. ANALYSIS

By the end of the thermal protocol, the Montania® 483 Real-Time PCR Instrument software automatically calculates the baseline cycles and the threshold.

Example of an amplification curve is given in Fig. 4.

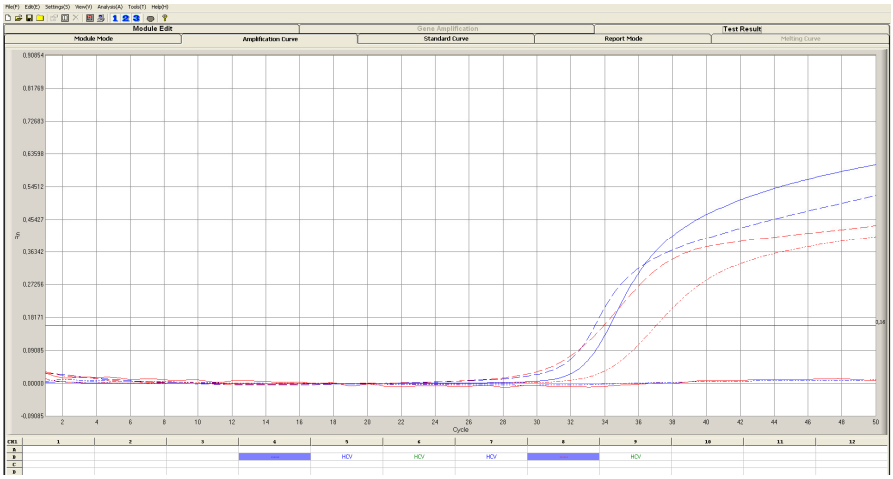

Fig. 4: Amplification Curve of a Bosphore® HCV Genotyping Kit v1

Analysis of the results should be performed by trained personnel who have received the required training for analysing Real-Time PCR data. We recommend that the test results must be evaluated by an expert clinician, taking the patient's clinical findings and the results of other tests into consideration.

All analysis is done automatically in routine use. However, when the trained personnel, who have received the required training from manufacturer, consider it as necessary, the system allows pulling down the threshold as much as possible in order to detect low positive samples. In this case, attention should be paid to keep the threshold line above the background.

Test results should not be reported unless there is amplification of the internal control in negative samples. Please contact the manufacturer if an impairment in the product's performance is observed (See the last page for contact information).

Qualitative results of the test are displayed on the "Report Mode" screen. A spread sheet containing the results of the unknown samples in each tube is shown in the "Test Result" column. The samples that cross the threshold in Channel 1 (FAM), Channel 2 (HEX) and Channel 3 (Cy5) are displayed with their positive/negative results, samples that do not cut the threshold are displayed as "No Ct". These samples are regarded as negative or having a viral load below the detection limit of the assay. For these undetectable samples, the Cy5 data of the internal control should also be checked to avoid false negative results.

The table below shows the possible results and their interpretations:

Channel/Detection Mix No	FAM	HEX	Cy5	Result
1	+	-	-	HCV 4
1	-	+	-	HCV 1b
1	-	-	-	Test must be repeated
2	+	-	-	HCV 1a
2	-	+	-	HCV 2
2	-	-	-	Test must be repeated
3	none	+	-	HCV 3
3	none	-	-	Test must be repeated
4	+	none	-	HCV 1 (1c,d,e,f,g,h,l,j,k)
4	-	none	-	Test must be repeated

11. TROUBLESHOOTING

Please contact the manufacturer in case of a problem during a run.

Late or no signal from the FAM or HEX filter

Wrong thermal protocol is chosen	Make sure that the correct thermal protocol is chosen.

Late or no signal from the Positive Control

Deterioration of the positive control or other kit components	Do not use positive controls or other kit components after their expiration date. Follow the instructions for the storage of kit components (See Section 3. Storage)

No signal from the internal control

Deterioration of the internal control or detection mix	Follow the instructions for the storage of kit components (See Section 3. Storage).
PCR inhibition	Make sure that you use the recommended RNA isolation method (See 9.3 RNA isolation).
RNA lost during isolation	Make sure that you use the recommended RNA isolation method (See 9.3 RNA isolation).

Signal from FAM or HEX Filter in the Negative Control

Contamination	Use filter-tips. Repeat PCR with new kit components.

The Threshold is Above Low Signals

The threshold should be manually adjusted	Using the mouse pull the threshold down until it cuts the low signals. Avoid the background and the signal from negative control.

12. SPECIFICATIONS

12.1. Sensitivity

Analytical sensitivity may be expressed as the limit of detection: i.e. the smallest amount of the target marker that can be precisely detected. The detection limit of an individual analytical procedure is the lowest amount of nucleic acid in a sample which can be detected but not necessarily quantitated as an exact value. The analytical sensitivity or detection limit for NAT assays is expressed by the 95% positive cut-off value.

The analytical detection limit for Bosphore® HCV Genotyping Kit v1 was found to be $1x10^2$ IU/ml (p=0.05). The sensitivity was determined using serial dilutions of RNA calibrated with the WHO International Standard for HCV RNA NAT assays, (NIBSC Code 06/100). The dilutions were tested in different runs in replicates. The results were analyzed by probit method.

12.2 Genotype Detection

Efficiency of detecting and quantitating different genotypes were ensured both by sequence comparison analysis and a Real-Time PCR assay using Worldwide HCV Performance Panel WWHV302(M) (Seracare). The following genotypes were tested and found positive:

Panel Member	Genotype	HCV(FAM)
1	1b	+
2	1a	+
3	1b	+
4	2a/2c	+
6	3b	+
8	3a	+
10	4	+
11	4	+
12	5a	+
14	6a	+

12.3. Cross-Reactivity

To eliminate potential cross-reactivity, both assay design evidence and experimental studies were employed. Primer and probe sequences were checked for possible homology to other known pathogen sequences by sequence comparison analysis using database alignment. Samples of HIV, HDV, HBV with known high positivity were tested, and found negative.

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14. SYMBOLS

Use by

Lot/Batch

Catalog number

Temperature limitation

Caution, consult accompanying documents

Manufacturer

In Vitro Diagnostic Medical Device

15. CONTACT INFORMATION

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